A NEW METHOD OF PRODUCING TWINS, TRIPLETS AND QUADRUPLETS IN ARBACIA PUNCTULATA, AND THEIR DEVELOPMENT

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Method

The simplest method of obtaining twins experimentally is from the first two blastomeres of a developing egg. In the early classical experiments of Driesch (1891, 1892), the isolated blastomeres of the sea-urchin egg were obtained by shaking. Later, he (1900) and most investigators since, have made use of Herbst's (1900) discovery that in Ca-free sea water, the ectoplasmic layer which binds the blastomeres together is dissolved and the blastomeres fall apart spontaneously or with slight shaking. Dilute sea water, heat, and the lack of certain ions (other than Ca) in the sea water also cause the blastomeres to separate (Driesch, 1892, 1906; Loeb, 1909). Some recent investigators have used free-hand cutting with glass needles, and others have used this method in combination with Ca-free sea water. These methods are tedious and the paired blastomeres must be isolated at an early stage; moreover, as pointed out by others, the procedure is injurious to the eggs, so that many of the separated blastomeres do not reach the blastula stage, and comparatively few the pluteus; it is therefore difficult to evaluate the results.

A new and much more efficient method of obtaining large quantities of twins and quadruplets from isolated blastomeres of the *Arbacia* egg was found in the course of some other experiments. No extensive work has been done, but the method is so simple and the general results so striking that it seems worth while to put them on record. The procedure is as follows. The eggs are fertilized, and the fertilization membranes removed by shaking two minutes later. The eggs are then allowed to develop in ordinary sea water till just after first cleavage (53 minutes after fertilization at 23° C.). Then the eggs are placed in hypertonic sea water, made either by boiling sea water to half its volume, or by adding 30 grams of NaCl per liter of sea water (same solution as used by me (1936) for parthenogenesis). The eggs are left in this solution 5–10 minutes, then returned to sea water. The first two blastomeres are nicely separated and develop independently forming a pair of twins,

joined by a thin cord or film. They swim at first in pairs, and can be easily isolated at this stage and their development followed; they soon become entirely independent of each other. By this method, large quantities can be obtained, and the pairs are isolated at a much later stage than by previous methods. In this way, eggs which have been injured by shaking or by the changed concentration of the sea water are eliminated, and since only very healthy-looking, active swimmers need be chosen, their fate seems a good criterion of the final development of isolated blastomeres. The older idea of the totipotency of the blastomeres of the echinoderm egg has been questioned by some more recent investigators. Of course, one might claim that all blastomeres lacking certain materials or cleaved in some particular plane died before reaching the blastula stage, but this seems to me improbable.

Twins

The production of the twins is apparently due to the effect of the hypertonic sea water on the ectoplasmic layer which binds the cells together. The hypertonic sea water causes the ectoplasmic layer to become very thick (Cf. Photograph 1 with 2, the control), and this is true for the eggs at any stage, and whether with or without fertilization membranes. At the time of cleavage, the thickened layer forms a heavy coat around each blastomere (Photograph 1). When the eggs are returned from the hypertonic sea water to ordinary sea water, the ectoplasmic layer swells and spreads out, becoming thin and gelatinous, and the two blastomeres are often widely separated with only a thin film between (Photograph 3). Any protoplasmic connection is soon broken. Apparently then, blastomeres can be separated both by a thickening of the ectoplasmic layer caused by hypertonic sea water, and by a dissolution of the layer caused by absence of calcium or by hypotonic sea water. It may be, however, that in the present case, the change from the hypertonic solution to the isotonic sea water is responsible for the effect rather than the hypertonic solution itself. If the fertilization membranes have not been removed, the two blastomeres do not develop separately, but apparently are so pressed together that they develop as one embryo.

Each blastomere divides into two equal cells at the same time that the whole egg divides into four (Photographs 4, 5), and into four equal cells when the whole egg divides into eight (Photograph 6). No critical work has been done on the micromeres, but in some cases two were present after the next cleavage, and in some cases they were not observed. By subsequent cleavages, each original blastomere gives rise to a spherical blastula, quite normal in appearance, but of half the normal volume

and with half the normal number of cells. The blastula is not the open type like the half blastula of *Parechinus*, but is a closed sphere like that of *Sphaerechinus* (Driesch). The half blastulae become free-swimming at the same time as the whole blastulae (Photographs 7–9). The gelatinous material remaining from the ectoplasmic layer still holds the pairs together even after they become actively free-swimming (Photograph 9), and it is at this time that one isolates the twins to follow their history.

An hour or so after becoming free-swimming, the twins separate and swim independently. The later development is slower than that of the whole blastula; the pluteus stage is reached on the third day instead of the second. In some cases in which individual pairs were isolated, both blastulae developed into plutei, perfect in structure, but smaller than normal (Photograph 10, *A, B*). In some cases, one developed into a perfect pluteus, the other was underdeveloped or abnormal (Photograph 11), and in some cases both developed abnormally or remained in an arrested state of development (Photograph 12).[1] Those which are designated as underdeveloped and abnormal were large blastulae ("Dauerblastulae"?) and gastrulae which did not subsequently become plutei, and plutei with abnormal skeleton or arms. When isolated in lots of 5 or 10 pairs of twins, in some batches few became perfect plutei (Photograph

PLATE I

Twins, Early Development

The photographs were taken with a Leica camera, of living eggs and embryos of *Arbacia punctulata*, and are not retouched; the swimmers were narcotized with chloretone. Most of the photographs were taken with a water immersion (× 40) lens and a 10 × ocular and are magnified about 250 × as presented. Photographs 10–13 and 30 were taken with a 16 mm. (× 10) lens and a 20 × ocular and are magnified about 125 ×; Photograph 7, same optical system but now magnified 250 ×. Photographs 14–16 and 24, 25, 38, 41 were taken with a 16 mm. (× 10) lens and a 10 × ocular and are magnified about 60 × as presented.

PHOTOGRAPH 1. Eggs in hypertonic sea water at the time of first cleavage. Note thickened ectoplasmic layer.

PHOTOGRAPH 2. Control; normal eggs in sea water with normal ectoplasmic layer.

PHOTOGRAPH 3. Eggs 7 minutes after return to sea water from hypertonic sea water. Two blastomeres separated with thin film between. One and one-quarter hours after fertilization.

PHOTOGRAPHS 4, 5. Each blastomere 2-cell. One and three-quarters hours after fertilization.

PHOTOGRAPH 6. Each blastomere 4-cell. Two hours after fertilization.

PHOTOGRAPH 7. Each blastomere 16-cell. Three hours after fertilization.

PHOTOGRAPH 8. Each blastomere an early blastula. A whole blastula to right. Four hours after fertilization.

PHOTOGRAPH 9. Twin blastulae just before becoming free-swimming. Five and one-half hours after fertilization.

[1] These three classes occurred in approximately equal numbers in my experiments.

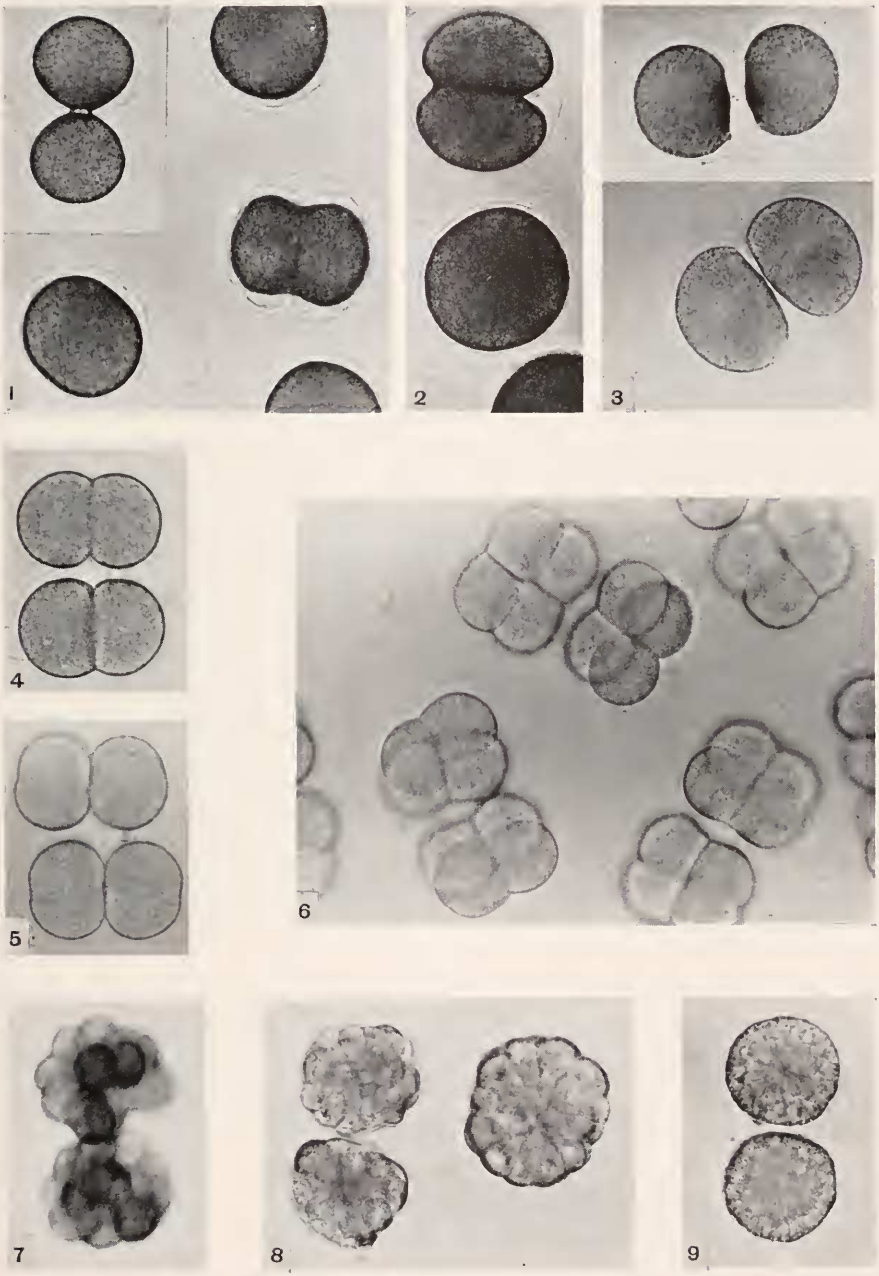


PLATE I

15), in other batches, many. In one batch all of the twenty (from 10 pairs) were perfect plutei except one which was a well-formed gastrula (Photograph 14). In such lots, one embryo may reach the pluteus stage a day before the others. As a control to the twins, some whole blastulae from the experimental lots were isolated at the same time in pairs and in lots of 20, and though they looked in fine condition, they gave rise to the same sort of abnormal forms as the twins but in a smaller proportion. A comparison of Photograph 13 with 11 and of Photograph 16 with 14 will show the similarity in the development of the whole and half blastulae. Even when normal blastulae are isolated in countable lots from *normal* cultures, usually one or two do not develop perfectly normally.

Since the results are so variable in different lots of the twins and since similar abnormalities occur also among the whole blastulae, it seems probable that the abnormal development is due to the experimental conditions (most likely to the moist chamber) and to innate peculiarities of the eggs, rather than to any lack of special materials separated off by cleavage planes. This variability in different experimental lots must be taken into account by investigators in this and related fields who, owing to the difficulties of operation, necessarily use small numbers of eggs, and often do not keep adequate controls. There is no evidence whatever of any proportion of the twins lacking gut-forming or skeleton-forming material and therefore remaining as blastulae. Permanent blastulae ("Dauernblastulae") do occur, but they also occur in large numbers in certain supposedly normal lots of developing whole eggs which have not been "ectodermised." And there is no evidence that the two twins are complementary so that the first two blastomeres represent the right and left sides of the embryo. Occasional one-armed plutei do

PLATE 11

Twins, Later Development

PHOTOGRAPH 10, *A*, *B*. Two twin plutei from the same egg, isolated as blastulae. Both perfect. Two days old.

PHOTOGRAPH 11. One twin perfect, one underdeveloped, from the same egg. Two days old.

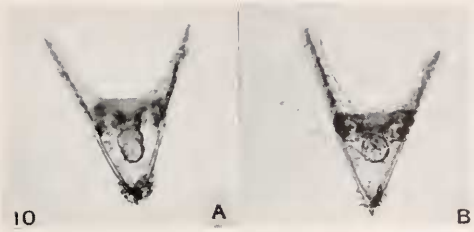
PHOTOGRAPH 12. Both twins underdeveloped or abnormal. Two days old.

PHOTOGRAPH 13. One perfect pluteus, one abnormal; from two whole blastulae, isolated from same dish as the above sets of twins. One day old. Cf. with Photograph 11.

PHOTOGRAPH 14. Plutei from 10 pairs of twin blastulae. All perfect except one, a good gastrula. Three days old.

PHOTOGRAPH 15. Another set of 5 pairs from another culture. One perfect pluteus, one nearly perfect, 6 gastrulae with skeleton, 2 blastulae without skeleton. Three days old.

PHOTOGRAPH 16. A lot of 21 whole embryos isolated at the blastula stage from experimental culture (as a control to twins). Note several imperfect or underdeveloped. Two days old. Cf. with Photographs 14, 15.



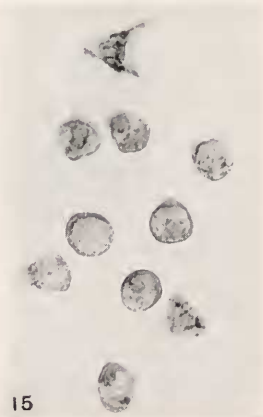


PLATE II

occur, but these have been found also in very different experiments. Nor does there seem to be any dorsal or ventral predominance in either of the developing blastomeres. These conclusions as to the early organization of the echinoderm egg, and especially of the *Arbacia* egg, are in general opposed to those of Plough (1927, 1929) and Hörstadius 1928, 1936, 1939), and are more in line with the original ideas of Driesch and those expressed by Wilson in "The Cell," and the more recent ones of von Ubisch (1936, 1938). It may be seen from Photographs 10, *A*, *B* and 14 that the first two blastomeres can and under optimum conditions, probably do develop into normal small plutei. The first cleavage plane must either divide the egg into two exactly similar parts, or else each of the first two blastomeres must be able to regenerate any material necessary for a normal pluteus which has been segregated from its mate by the first cleavage plane. It might be pointed out that the eggs of different species of sea urchin are quite different, as shown by their appearance and by their stratification with centrifugal force. Some may be more highly organized than others, and materials may be more localized. One has only to consider the pigment band in the *Paracentrotus* egg, which is not found in other species, and varies greatly in intensity in that species in different localities, and even in the same locality. This may be visible evidence of a greater localization of materials in this egg than in the *Arbacia* egg. The first two blastomeres of *Arbacia punctulata* are certainly totipotent, whatever they may be in other sea urchins.

Quadruplets

If the *Arbacia* eggs from which fertilization membranes have been removed are placed in the hypertonic sea water just after second cleav-

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PLATE III

Quadruplets

PHOTOGRAPH 17. Eggs in hypertonic sea water just after second cleavage. Note thickened ectoplasmic layer.

PHOTOGRAPH 18. Eggs 13 minutes after return to sea water from hypertonic sea water. Four blastomeres separated with film between. One and three-quarters hours after fertilization.

PHOTOGRAPH 19. Each blastomere 2-cell. Two hours after fertilization.

PHOTOGRAPH 20. Asynchronous first cleavage of four separated blastomeres. Two hours after fertilization.

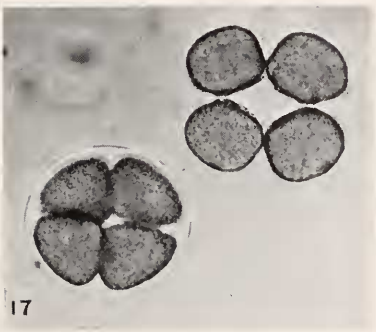
PHOTOGRAPH 21. Each blastomere 4-cell. Two and three-quarters hours after fertilization.

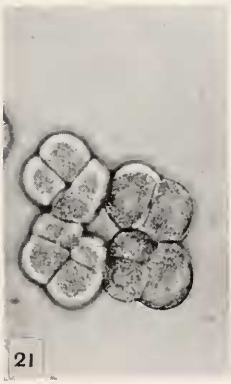
PHOTOGRAPH 22. Quadruplets in later cleavage. A pair of twins above, and two whole eggs below. Three and one-quarter hours after fertilization.

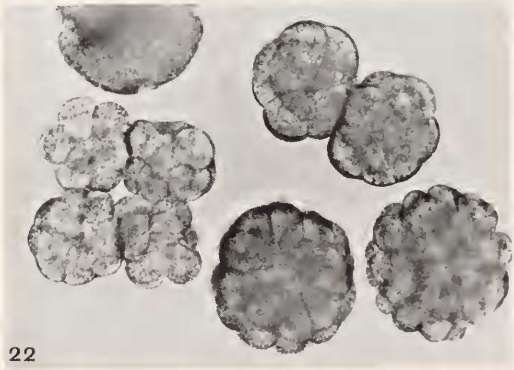
PHOTOGRAPH 23. Quadruplets just before becoming free-swimming. Note film still holding them together. Five hours after fertilization.

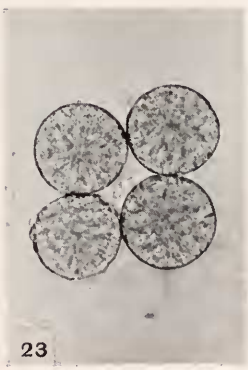
PHOTOGRAPH 24. Quadruplets from same egg, all perfect plutei. Two days old.

PHOTOGRAPH 25. Two sets of quadruplets, all imperfect plutei with skeleton. Three days old.









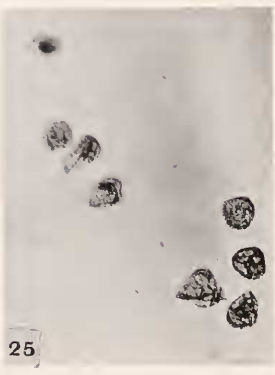


PLATE III

age, the ectoplasmic layer between the four cells becomes thickened (Photograph 17). When, after 5–10 minutes, they are returned to ordinary sea water, the four cells become separated but linked together by a film of gelatinous material (Photograph 18). Each blastomere cleaves at the same time as the whole egg, though one cell may be in advance of the others (Photographs 19, 20). Each blastomere passes through subsequent cleavages independently, until four small (closed) blastulae are formed (Photographs 21, 22, 23). These swim together at first in quartets, and it is at this stage that they are isolated; they soon break apart and become independent swimmers. The quadruplets cleave at the same time and become free-swimming at the same time as the twins and whole eggs. Each one has therefore only one quarter the normal number of cells. Some of the sets of four blastomeres give rise to plutei all absolutely normal except in size (Photograph 24). There is some individual variation in size but this is true also of individuals coming from whole eggs. Some quartets give rise to plutei somewhat abnormal or underdeveloped but usually with skeletons (Photograph 25). The plutei from the quarter blastulae are formed more slowly than from whole blastulae.

The development of the quadruplets gives no indication that any one of them lacks any particular material segregated by the first or second cleavage planes. Nor is there any evidence of any dominance of a right or left side, or of a dorsal or ventral part. Each of the quadruplets is in general like its mates, but some lots develop much better than others, owing probably to experimental conditions or to innate differences in vitality. The second cleavage plane, as well as the first, either divides the egg into exactly similar parts, or else each blastomere regenerates any material segregated into the others by the cleavage planes.

The quartets would serve as excellent material for a more critical study since it is quite possible to tell the exact relation of each member of the quartet to the first and second cleavage planes; the second plane is

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PLATE IV

Triplets and Fused Twins

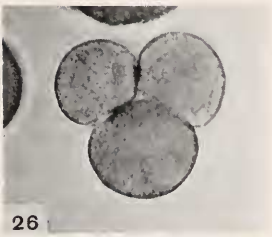
PHOTOGRAPHS 26, 27. Beginning of triplets. Two blastomeres at 4-cell stage remain separate, and other two remain together or fuse. One and three-quarters hours after fertilization.

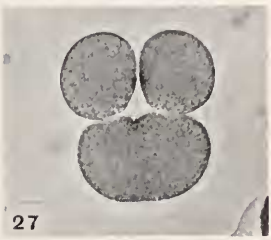
PHOTOGRAPH 28. Fusion of two of quadruplets at a later stage, forming triplets. Three and one-quarter hours after fertilization.

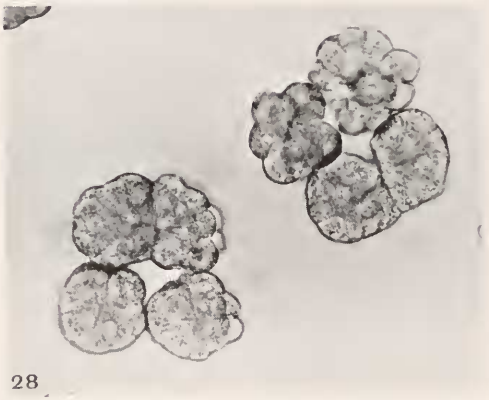
PHOTOGRAPH 29. Triplets in blastula stage. Whole blastula to left. Four and one-quarter hours after fertilization.

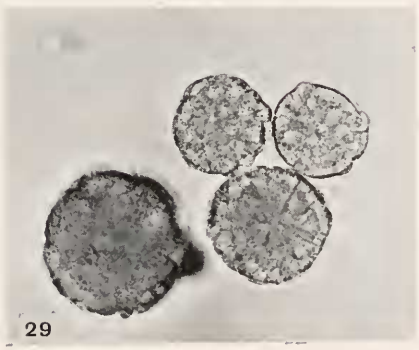
PHOTOGRAPH 30. Triplets. One large perfect pluteus, one small pluteus slightly imperfect, one small underdeveloped pluteus with skeleton. Two days old.

PHOTOGRAPH 31. Blastulae from partially fused twins (2 center ones). A pair of twins to left, and a whole blastula to right. Four hours after fertilization.









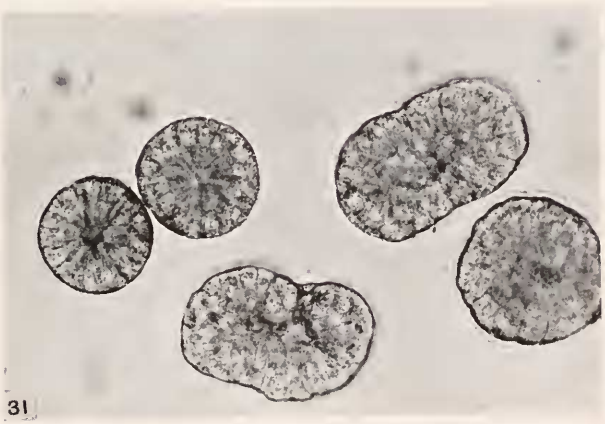


PLATE IV

longer than the first (Photograph 17). This is due to the fact that the pair of cells made by the first cleavage plane is more widely separated at the four-cell stage than the pair made by the second cleavage plane. It is probably on this account that twins as well as quadruplets occur in quantity just after the second cleavage (after treatment with hypertonic sea water). There was no difference in development of these twins, after treatment at the four-cell stage (Photograph 22), and those obtained after treatment at the two-cell stage.

Attempts to separate the eight blastomeres at the next cleavage were unsuccessful; the cells always came together again and developed as a whole. But this method might be used successfully for the eight-cell stage in combination with some other treatment.

Triplets

Triplets are obtained from the quadruplet sets in which only two of the cells remain separate, and the other two develop as a whole (Photographs 26, 27); or two of the four which have started to develop independently, later on become fused while the other two remain distinct (Photograph 28). The triplets, consisting of one large and two small parts, develop into free-swimming blastulae, and they can be isolated at this stage (Photograph 29). These develop into one large pluteus (corresponding to one twin) and two smaller ones (corresponding to two

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PLATE V

Twins Obtained by Centrifugal Force

PHOTOGRAPH 32. Egg after having been centrifuged 40 minutes after fertilization at about 10,000 × g for 6 minutes. Note the ectoplasmic layer lying as a crescent in the broken fertilization membrane.

PHOTOGRAPH 33. Two blastomeres after separation by centrifugal force. Centrifuged 1 hour after fertilization, photographed immediately after removal from centrifuge.

PHOTOGRAPH 34. Same pair, each blastomere 2-cell. Two hours after fertilization.

PHOTOGRAPH 35. Same pair, each blastomere 4-cell. Three hours after fertilization.

PHOTOGRAPH 36. Same pair, each blastomere 8-cell. Three and one-quarter hours after fertilization.

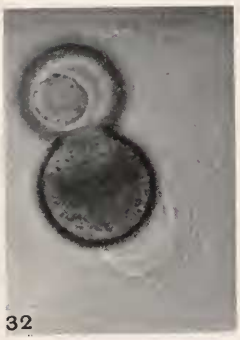
PHOTOGRAPH 37. Twin blastulae just before becoming free-swimming. Six hours after fertilization.

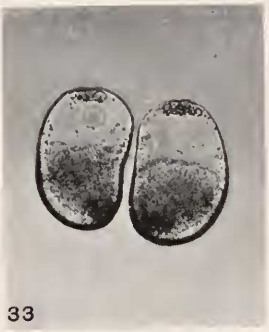
PHOTOGRAPH 38. Two twin plutei from the same egg, isolated as blastulae. Both perfect. Three days old.

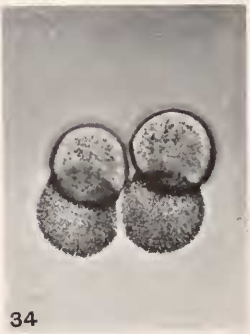
PHOTOGRAPH 39. Twin blastulae inside fertilization membrane. Six hours after fertilization.

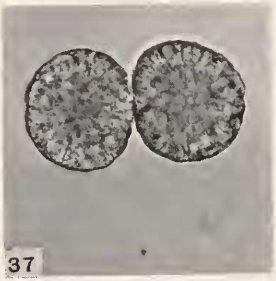
PHOTOGRAPH 40. Blastula from partially fused twins. Six hours after fertilization.

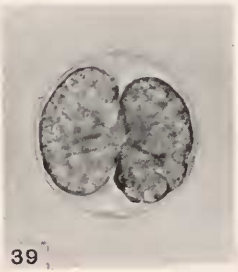
PHOTOGRAPH 41. Eggs in two-cell stage centrifuged on the centrifuge microscope, photographed while rotating.

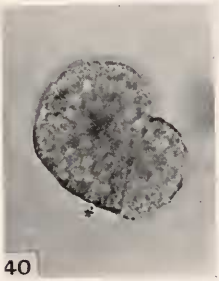












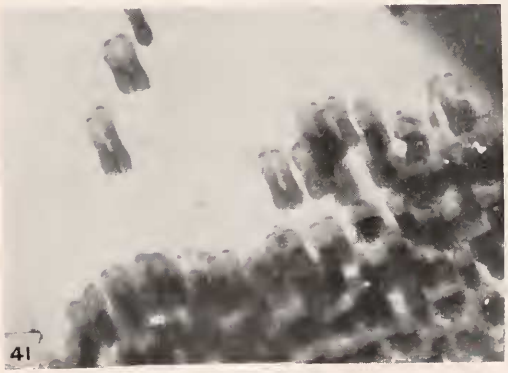


PLATE V

quadruplets) all of which may be more or less perfect, varying in different lots (Photograph 30).

Fused Twins

After the first two blastomeres have been separated and have started to develop independently, they may again fuse. All degrees of fusion occur, and these fused forms become free-swimming. These may be seen in Photograph 31 together with a pair of twins and a whole blastula. Their development has not been followed. Many of the more thoroughly fused twins are probably indistinguishable from normal whole blastulae, and this may partly explain the unexpectedly large number of imperfect forms developing in the controls to the twins (Photograph 16).

Twins Obtained by Centrifugal Force

Another entirely different method of obtaining twins was discovered several years ago for *Parechinus microtuberculatus* (E. B. Harvey, 1935). It is by centrifugal force, and this method is concerned also with the ectoplasmic layer. This layer can be centrifuged off from the surface of the egg at any stage of development (E. B. Harvey, 1934). When centrifuged off just after first cleavage, the two blastomeres are separated and may develop independently. The same phenomenon can be observed also in the *Arbacia* egg. The force used was about 10,000 $\times$ g. for 6 minutes. In Photograph 32, the ectoplasmic layer is seen as a crescent inside the broken fertilization membrane. Photograph 41 shows the eggs in the two-cell stage as they appear during centrifugation on the centrifuge-microscope. In Photograph 33, the two blastomeres are separated, and the independent development of the two blastomeres into two perfect plutei is shown in Photographs 34 to 38. The blastomeres remain attached until after the blastulae have become free-swimming and they can be isolated at this stage. In these cases the fertilization membrane had been removed before centrifuging, by shaking. The two blastomeres may also develop independently inside the fertilization membrane (Photograph 39). They may also fuse again after having been separated (Photograph 40). This method of obtaining twins is neither as simple nor as efficient as the one described in the earlier part of the paper.

Summary

1. Twins, triplets, and quadruplets of *Arbacia punctulata* are obtained by treating the eggs just after first and second cleavage with a hypertonic salt solution, which thickens the ectoplasmic layer; the blasto-

meres separate when returned to sea water. The blastomeres develop independently but attached to each other until they become free-swimming blastulae, when they can be isolated in pairs or quartets.

2. Twins, triplets and quadruplets from a single egg may all develop into perfect dwarf plutei.

3. There is no indication of any differences among the twins, triplets, and quadruplets of a set (i.e. from one egg) caused by the segregation of any special organ-forming materials by the first two cleavage planes, nor by a separation into a right and left half, or into an anterior and posterior part.

4. Differences and abnormalities in the development of twins, triplets, and quadruplets are probably caused by experimental conditions or by differences in vitality of the original egg, since abnormalities of the same sort occur among the controls, and since some lots develop much better than others.

5. Twins may also be obtained by centrifuging the eggs just after first cleavage. The ectoplasmic layer is centrifuged off, and the two blastomeres develop independently but attached to each other until they become free-swimming blastulae. Both twins may form perfect dwarf plutei.

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